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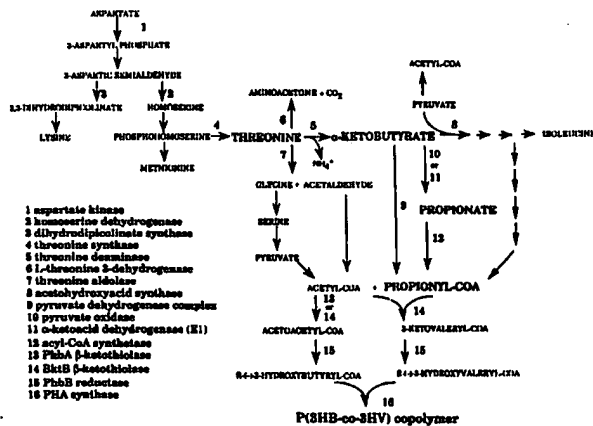
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification 6 :</b> <b>C12N 15/82, 15/54, 15/53, 15/52, 15/60,</b> <b>9/10, 5/10, A01H 5/00, C12N 1/21, C12P</b> <b>7/62</b>	<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 98/00557</b> <b>(43) International Publication Date:</b> <b>8 January 1998 (08.01.98)</b>
<b>(21) International Application Number:</b> <b>PCT/US97/08983</b> <b>(22) International Filing Date:</b> <b>28 May 1997 (28.05.97)</b> <b>(30) Priority Data:</b> <b>08/673,388</b> <b>28 June 1996 (28.06.96)</b> <b>US</b>		<b>(74) Agent:</b> <b>KAMMERER, Patricia, A.; Arnold, White &amp; Durkee,</b> <b>P.O. Box 4433, Houston, TX 77210 (US).</b>
<b>(71) Applicant:</b> <b>MONSANTO COMPANY [US/US]; 800 North</b> <b>Lindbergh Boulevard, St. Louis, MO 63167 (US).</b>		<b>(81) Designated States:</b> <b>AL, AM, AT, AU, AZ, BA, BB, BG, BR,</b> <b>BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE,</b> <b>GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR</b> <b>LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ,</b> <b>PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT,</b> <b>UA, UG, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW,</b> <b>SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD,</b> <b>RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES,</b> <b>FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent</b> <b>(BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD,</b> <b>TG).</b>
<b>(72) Inventors:</b> <b>GRUYS, Kenneth, James; 16534 Baxter Forest</b> <b>Ridge Drive, Chesterfield, MO 63005 (US). MITSKY, Tim-</b> <b>othy, Albert; 2262-A Rule Avenue, Maryland Heights, MO</b> <b>63043 (US). KISHORE, Ganesh, Murthy; 15354 Grantley</b> <b>Drive, Chesterfield, MO 63017 (US). SLATER, Steven,</b> <b>Charles; 819 H Foxsprings Drive, Chesterfield, MO 63017</b> <b>(US). PADGETTE, Stephen, Rogers; 553 Nantucket Pointe</b> <b>Drive, Grover, MO 63040 (US). STARK, David, Martin;</b> <b>318 West Manor Drive, Chesterfield, MO 63017 (US).</b> <b>HINCHEE, Maud, Ann, Wrightson; 2010 Medicine Bow</b> <b>Drive, Wildwood, MO 63011 (US). CLEMENTE, Thomas,</b> <b>Elmo; 2620 Winthrop Road, Lincoln, NE 68502 (US).</b> <b>CONNOR-WARD, Dannette, Vaudrilyn; 927 Hemingway</b> <b>Lane, St. Charles, MO 63304 (US). FEDELE, Mary,</b> <b>Jacqueline; 311 Bellerive Drive, Ballwin, MO 63011 (US).</b>		<b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
		<b>(88) Date of publication of the international search report:</b> <b>23 July 1998 (23.07.98)</b>

(54) Title: METHODS OF OPTIMIZING SUBSTRATE POOLS AND BIOSYNTHESIS OF POLY- $\beta$ -HYDROXYBUTYRATE-CO-POLY- $\beta$ -HYDROXYVALERATE IN BACTERIA AND PLANTS



**(57) Abstract**

Genes and methods for optimizing levels of substrates employed in the biosynthesis of copolymers of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) in plants and bacteria via manipulation of normal metabolic pathways using recombinant DNA techniques are provided. This is achieved through the use of a variety of wild-type and/or deregulated enzymes involved in the biosynthesis of aspartate family amino acids, and wild-type or deregulated forms of enzymes, such as threonine deaminase, involved in the conversion of threonine to P(3HB-co-3HV) copolymer endproduct. By these methods, enhanced levels of threonine,  $\alpha$ -ketobutyrate, propionate, propionyl-CoA,  $\beta$ -ketovaleryl-CoA, and  $\beta$ -hydroxyvaleryl-CoA are produced. Also provided are methods for the biological production of P(3HB-co-3HV) copolymers in plants and bacteria utilizing propionyl-CoA produced through a variety of engineered metabolic pathways. Introduction into plants and bacteria of an appropriate  $\beta$ -ketothiolase,  $\beta$ -ketoacyl-CoA reductase, and PHA synthase, alone or in combination with various enzymes involved in aspartate family amino acid biosynthesis and the conversion of threonine to PHA copolymer precursors, will permit these organisms to produce P(3HB-co-3HV) copolymers.

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# INTERNATIONAL SEARCH REPORT

Intern    al Application No <b>PCT/US 97/08983</b>					
<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC 6    C12N15/82    C12N15/54    C12N15/53    C12N15/52    C12N15/60 C12N9/10    C12N5/10    A01H5/00    C12N1/21    C12P7/62					
According to International Patent Classification (IPC) or to both national classification and IPC					
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) IPC 6    C12N    C12P    A01H					
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched					
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)					
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>					
Category *	Citation of document, with indication, where appropriate, of the relevant passages				Relevant to claim No.
X	TAILLON, B., ET AL.: "Analysis of the functional domains of biosynthetic threonine deaminase by comparison of the amino acid sequences of the three wild-type alleles to the amino acid sequence of the biodegradative threonine deaminase" GENE, vol. 63, 1988, pages 245-252, XP002045219 see page 249 - page 250 <div style="text-align: center;">---</div> <div style="text-align: center;">-/--</div>				26,28
<div style="display: flex; justify-content: space-between;"> <span><input checked="" type="checkbox"/> Further documents are listed in the continuation of box C.</span> <span><input checked="" type="checkbox"/> Patent family members are listed in annex.</span> </div>					
<div style="display: flex;"> <div style="flex: 1;"> <p>* Special categories of cited documents:</p> <p>*A* document defining the general state of the art which is not considered to be of particular relevance</p> <p>*E* earlier document but published on or after the international filing date</p> <p>*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>*O* document referring to an oral disclosure, use, exhibition or other means</p> <p>*P* document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="flex: 1;"> <p>*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>*A* document member of the same patent family</p> </div> </div>					
Date of the actual completion of the international search  <div style="text-align: center;">27 March 1998</div>			Date of mailing of the international search report  <div style="text-align: center;">1 0 -06- 1998</div>		
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016			Authorized officer  <div style="text-align: center;">Maddox, A</div>		

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/08983

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Intern: al Application No  
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A	MOURAD, G., ET AL.: "L-O-methylthreonine-resistant mutant of Arabidopsis defective in isoleucine feedback regulation" PLANT PHYSIOLOGY, vol. 107, 1995, pages 43- 52, XP002045223 cited in the application see page 51, left-hand column ---	3-8
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International Application No  
PCT/US 97/08983

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	<p>NAWRATH, C., ET AL.: "Targeting of the polyhydroxybutyrate biosynthetic pathway to the plastids of Arabidopsis thaliana results in high levels of polymer accumulation" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol. 91, December 1994, WASHINGTON US, pages 12760-12764, XP002045228 see the whole document -----</p>	1-20

# INTERNATIONAL SEARCH REPORT

In ternational application No.  
PCT/US 97/08983

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.



## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

## 1. Claims: 1-30, 40.

Plants and bacterium producing P(3HB-co-3HV) copolymer, comprising introduced DNA for the enzymes beta-ketothiolase(s) capable of synthesising both acetoacetyl-CoA and beta-ketovaleryl-CoA, a beta-ketoacyl-CoA reductase capable of reducing said intermediates to beta-hydroxybutyryl-CoA and beta-hydroxyvaleryl-CoA respectively, and a PHA synthase that incorporates both of said hydroxyacyl compounds into the P(3HB-co-3HV) copolymer, optionally containing a wild type or deregulated threonine deaminase, a method using said plants for the production of said copolymer, as well as the beta-ketothiolase and threonine deaminase(DNA molecules) used in said plants and bacterium.

## 2. Claims: 31-35

Plants producing P(3HB) homopolymer in their seeds, comprising introduced DNA encoding the enzymes beta-ketothiolase capable of producing acetoacetyl-CoA, phbB, and phbC, each linked to a transit peptide for directing said enzymes to a plastid, and regulatory signals for seed expression. Method for the production of said homopolymer using said plants, as well as seeds containing it.

## 3. Claim : 36

Method for Agrobacterium mediated transformation of canola by inoculating a stem explant prepared by the removal of leaves and buds along the stem and removing 4-5 inches of stem below the flower buds, further cutting said 4-5 inches of stem into segments, subsequently culturing said explant in a basal-side down orientation, and regenerating a transformed plant from the transformed tissue obtained thereby.

## 4. Claims: 37-39

Method for the Agrobacterium mediated transformation of soybean by inoculating a region adjacent to the axillary bud, in an explant prepared from a cotyledon of soybean seedling, by incubating said seedling at 0-10 degrees celsius for at least 24 hours, removing the hypocotyl region from the region of about 0.2 to 1.5 cm below the cotyledonary node, splitting the remaining attached hypocotyl segment thereby also separating the cotyledons, removing the epicotyl from its cotyledon, and wounding the cotyledon in the region of the axillary bud. Selection and regeneration of plants from transformed tissue obtained following said inoculation, as well as the explant and soybean tissue resulting from said preparation.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/08983

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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